Human - CD64 - Ig

STATUS: This clone, which is currently producing large amounts of soluble protein in CHO DG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield & Morrison, 1991 J Exp Med (173) 1483
Jones et al, 1991 J Immunol. (147)
Tao et al, 1991 J Exp Med (173) 1025
Duncan & Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated Residues 235 and 237 in γ_4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me

Date

Scott M. Cain

A-2

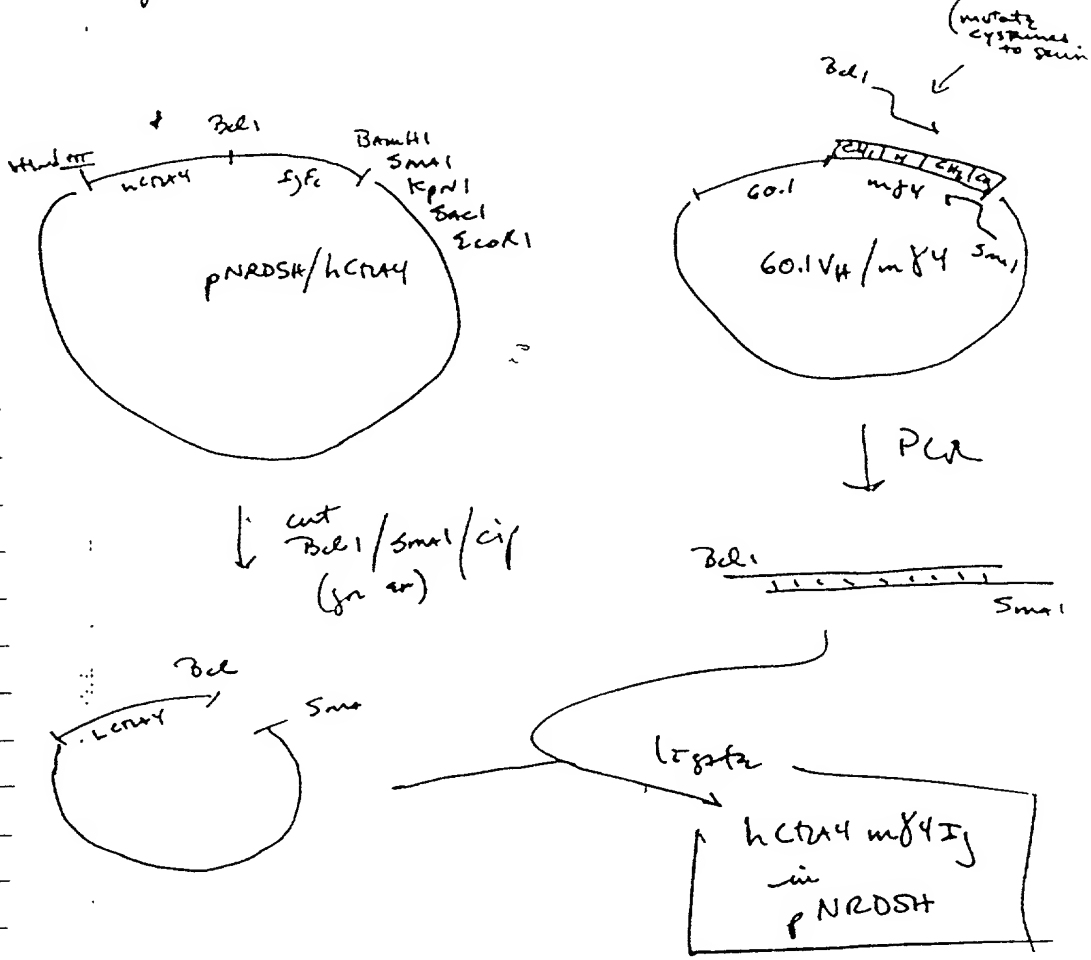
2 STRATEGIES will be USED:

hCγ4, mutagenesis of Fγ Fc

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the EXISTING γ1 H-CH_2-CH_3

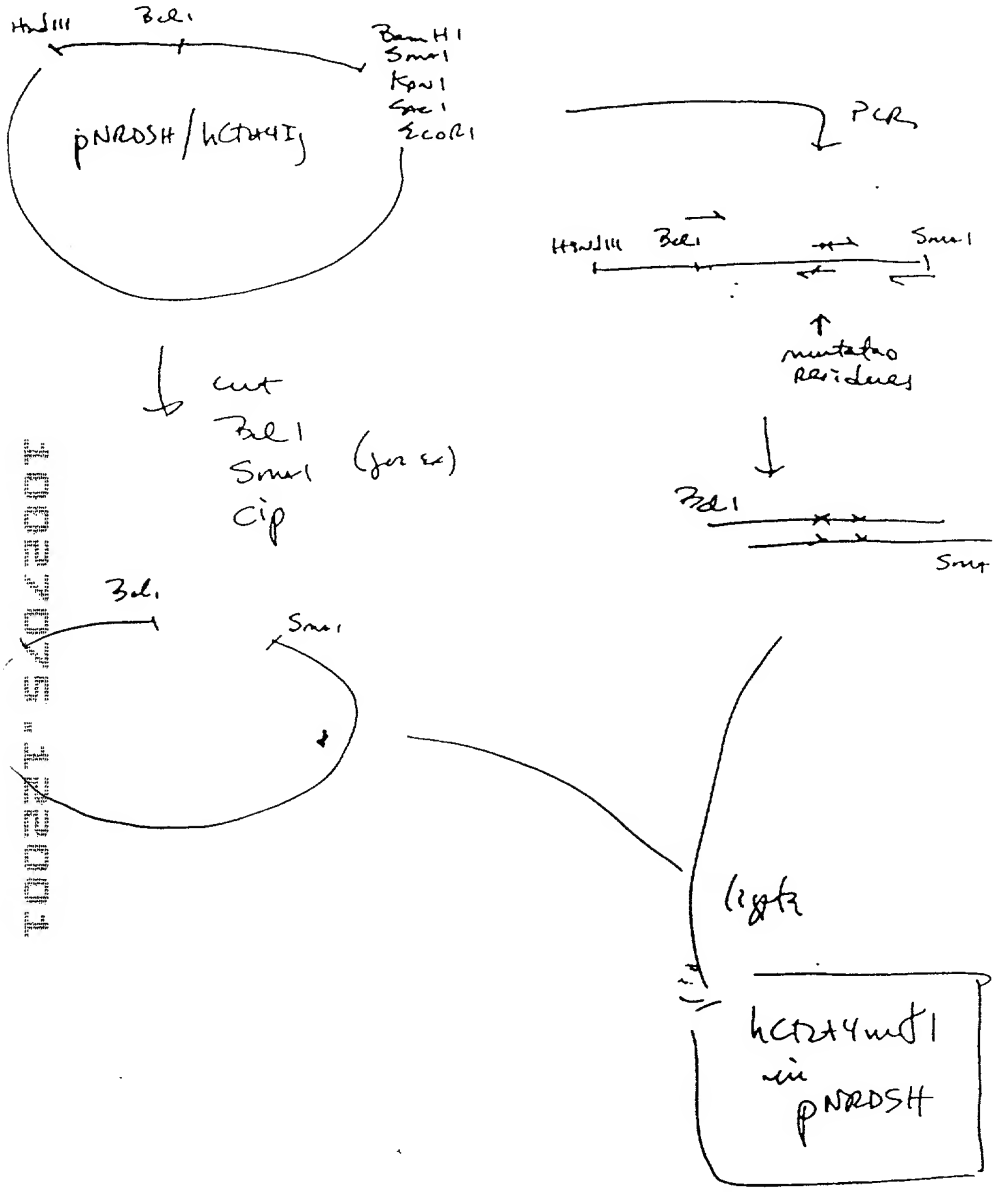
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

USE NESTED PCR to gener - a mutated 81 from hCTLA4Ig . Clone the mf1 back into hCTLA4 M pNRDSH :



For this clone mutate residues as follows:

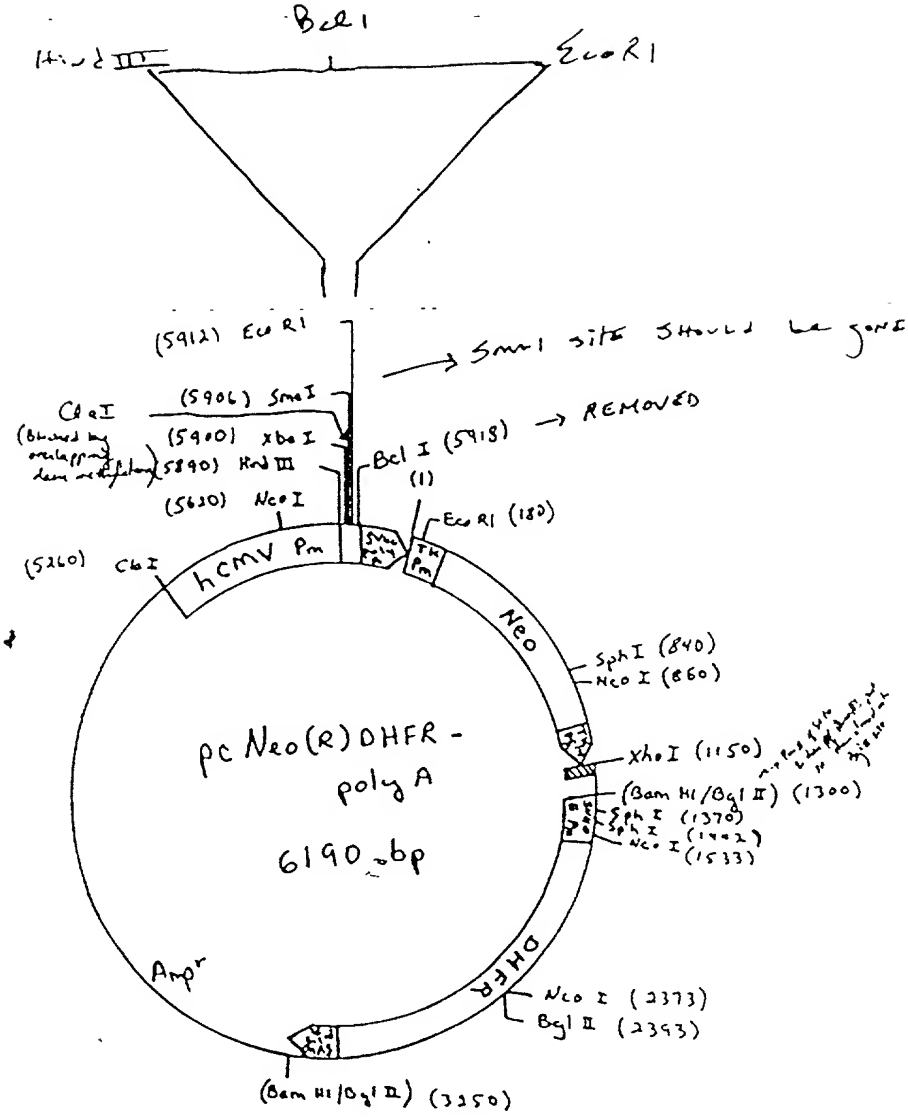
234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

A-4

Vector:



Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (1-1-92)

Read and understood by me

Date

primers for γFc mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S . K Y

BCL 1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

(G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

1st use this

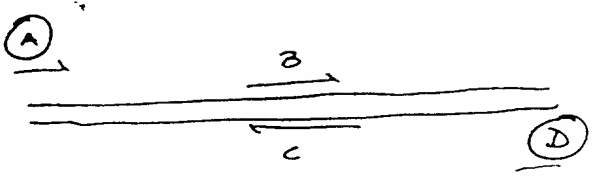
3' primer

if needed still have these

5' GCAGAG GAATTC GAGCTC GGTACCC GGGG ATCC

lock RI SacI KpnI XmaI BamHI

CCAGTGTGGGGACAGTGGGACCCGCTCTGCCTCCC 3'



5' primer ✓

(A): use Gary Gray's original 5' primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT

P P BglI a E P K S S D K T

CAC ACA TCT CCA CCG TCT CCA GGT AAA C — IgFc —

H T S P P S P G K

——→* — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-Bgl2 —

— T7 promoter

3' primer (D):

XmaI

BamHI SmaI KpnI SacI EcoRI

pSB72 MCS: 5' GGATCCCC GGGTACC GAGCTC GAATTC 3'

3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAG CTCGGTACCCGGGGATCC

lock

Read and understood by me

Date

1111 A-7

B and C:

L L G G P
CTC CTG GGG GGA CCG

A P E
(B) 5' CCATCTCTTCCTCAGCA CCT GAA

GCT GAA GGG GCT
GCG GAG ... GCC
GCA ... GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCC CGT

P S V F L F P
CCG TCA GTCTTC CTCTTCCCCC 3'

GGCAGT CAG AAGGAGAAGGGGG G GTTTT GGG 5' (C)

Oligonucleotide Requests

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Bennett

PROJECT CHARGED B7 & 9CT

DATE REQUESTED .

DATE REQUIRED (NO ASAP)

SEQUENCE NAME MUGAMMA4 - 5'

LENGTH 67

SEQUENCE:

pol I

5' G A G C A T T T C C T G A T C A G G A
G T C G A A A T A T G G T C C C C A T
C C C C A T C A T C C C C A G G T A A G
C C A A C C C 3'

Date

Read and understood by me

Grant Cam

Transient Expression of IgLh.CTLA4(3) Ig / EE12 A-8

→ 38

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

IDENTIFICATION		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4(#2)-γ1	1	2.12	1.77	↓
CTLA4-mγ4	2	14.88	3.23	
IgL CTLA4(#2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.9	35.54	↓

⊕ Controls 2.28 μg/ml 156 ng/ml

expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 binding. Assay run by Nancy Green.

Optical Density

	1 (unlabelled CTLA4-Ig)	2 (JC samples #1)	3 (#2)	4 (#3)	5 (#4)	6 (WT 6/16)	7 (7/13)	8 (WT 7/13)	9 (Avg A504)	10	11	12
50 μg/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
11.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
5.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(no x ... ligand ...)

As before the IgLhCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete the CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NSO lines.

→ Samples titrated serially 1:2 - in 50 μl vol

→ All sample wells contain 50 μl of 70 ng/ml CTLA4 Ig biotin

→ 43

Read and understood by me Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver

Serial No.: ~~09/227,595~~ Continuation of 09/227,595

Filed: ~~January 8, 1999~~ December 20, 2001

For: CTLA4-Cγ4 Fusion Proteins (as amended)

Attorney Docket No.: RPN-001

Group Art Unit: 1642

Examiner: Helms, L.

Assistant Commissioner for Patents
Washington, D.C. 20231

Certificate of ~~First Class Mailing~~ (37 CFR ~~1.8(a)~~ Under 1.10)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ "Express Mail to Addressee" in an envelope addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor for Megan E. Williams
~~Megan E. Williams~~
Registration No. 43,270
~~Attorney for Applicants~~

Mailing Label No. EL 833315914US

MEW

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

2. Prior to July 11, 1994, the invention described and claimed in the above-referenced application was completed in this country, as evidenced by the following:

A. Prior to July 11, 1994, we were in possession of a cloned CTLA4Ig molecule which was producing large amounts of active protein in CHO DG44 cells.

Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

Page A-2 of Appendix A describes amplification of the mutated γ4 Hinge-CH_2-CH_3 region and the cloning of the mutated γ4 into pNRDSH/hCTLA4 to replace the existing γ1 Hinge-CH_2-CH_3.

Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: _____ Signed: _____

Gary S. Gray

Date: October 4, 2001 Signed: _____

Jerry Carson

Date: _____ Signed: _____

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: _____ Signed: _____

Sandra Silver

HUMAN - CD44 : Ig

STATUS : This clone, which is currently producing large amounts of native protein in CHO DG44, is being methotrexate amplified.

RECENTLY focus was shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
Jung et al, 1991 J Immunol. (147)
Tao et al, 1991 J Exp Med (173) 1025
Duncan + Winter, 1988 Nature (332)

IT would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year (on the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated residues 235 and 237 in γ_4

see p 115, Book 1139 and //

see p 52, Book 1177 and //

Read and understood by me

Date

Scott M. Cain

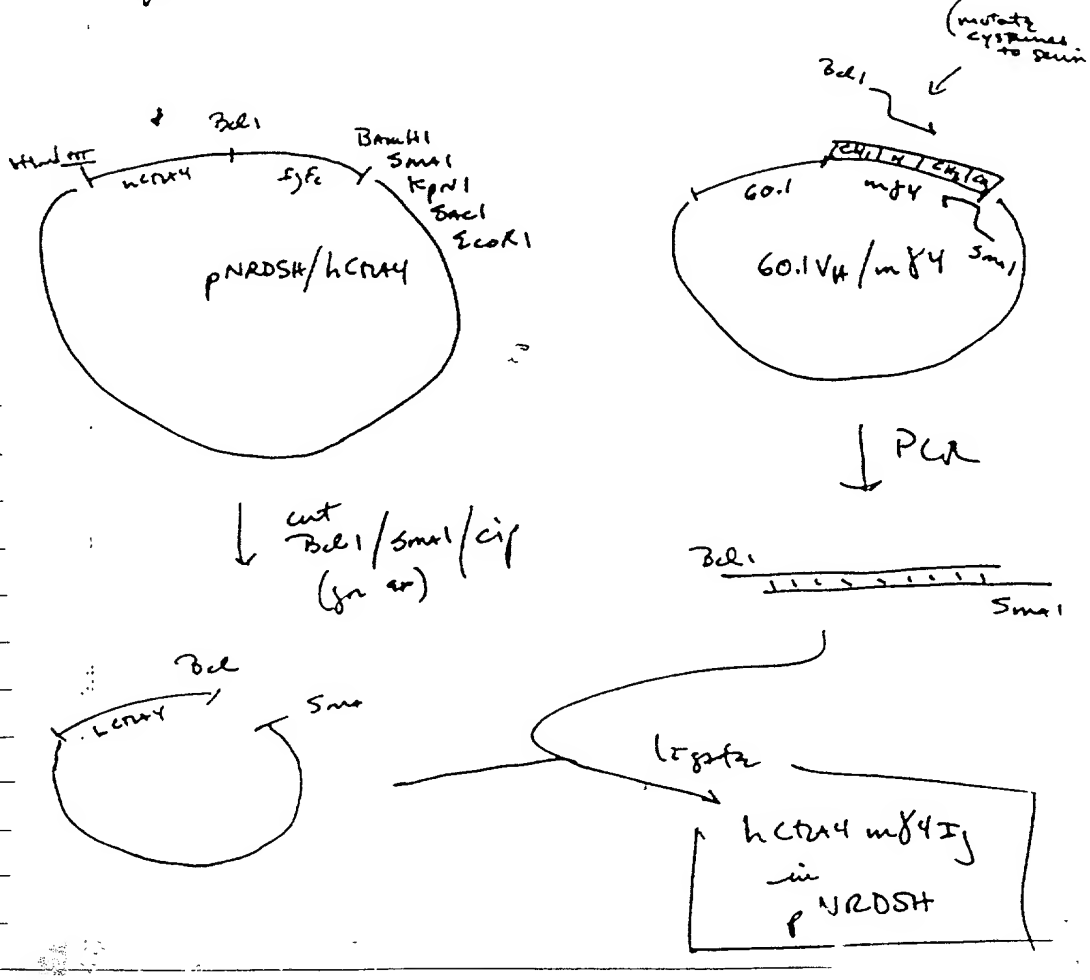
2 STRATEGIES will be USED:

hCγ4, mutagenesis of IgFc

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the existing γ_1 H-CH_2-CH_3

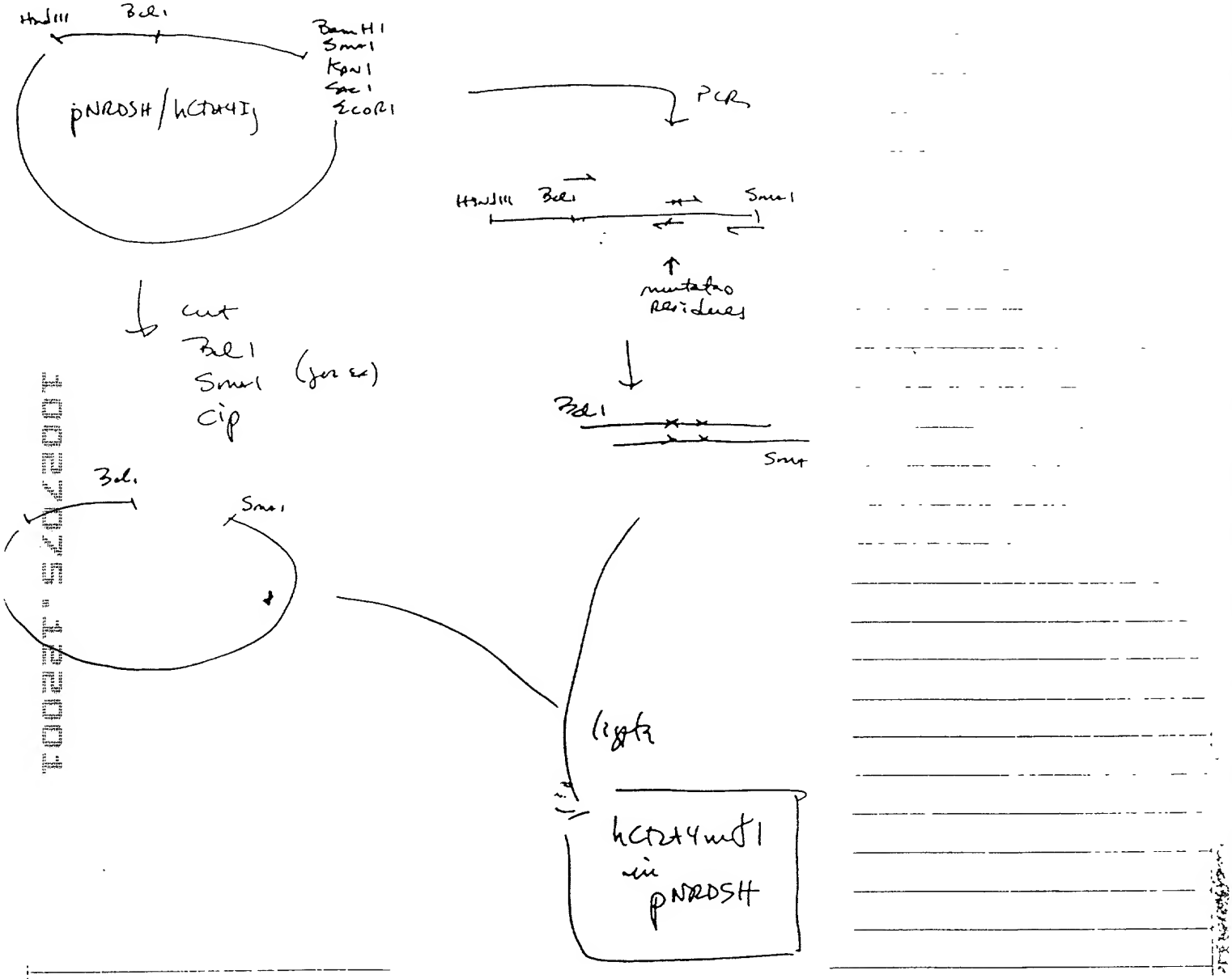
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

USE NESTED PCR to gener. a mutated 81 from hCD24 Ij. Clone the mf1 back into hCD24. M
pNRDSH:



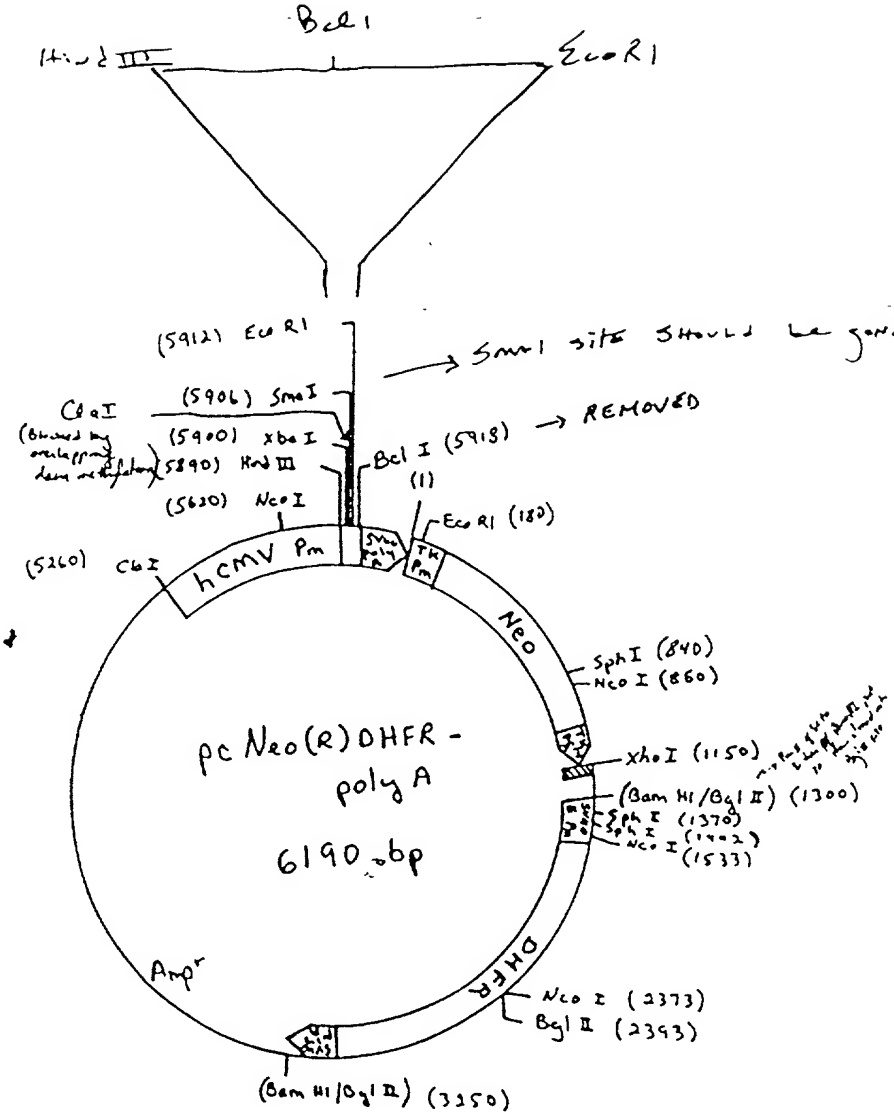
For this clone mutated residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



→ SmaI site should be gone

→ REMOVED

preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (1-9-92)

Read and understood by me

Date

primers for γFc mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) BCL1 E S K Y

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

(G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

1st use this

3' primer

if needed still have these

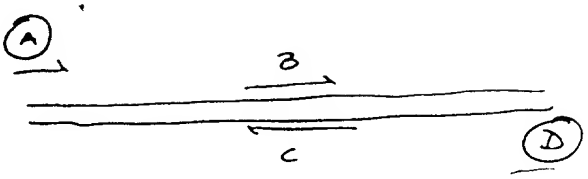
5' GCAGAG GAATTCGAGCTC GGTACCCGGGGATCC

lock RI SacI KpNI XmaI BamHI

CCAGTGTGGGGACAGTGGGACCCGCTCTGCCTCCC 3'

Read and understood by me [signature] Date

For [illegible]:



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT
P D BglI a E P K S S D K T
CAC ACA TCT CCA CCG TCT CCA GGT AAA C — IgF_c —
H T S P P S P G K

——— * — PvuII-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRV-BglI —

— T7 promoter

3' primer (D): XmaI

BamHI SmaI KpnI SacI EcoRI

pSR72 MCS: 5' GGATCCCC GGGTACC GAG CTC GA ATTC 3'

3' CCTAGGGG CCCATGG CTC GAG CTTAAG 5'

PRIMER:

5' GCAGAG GAATTCGAG CTC GGTACCCGGGGATCC

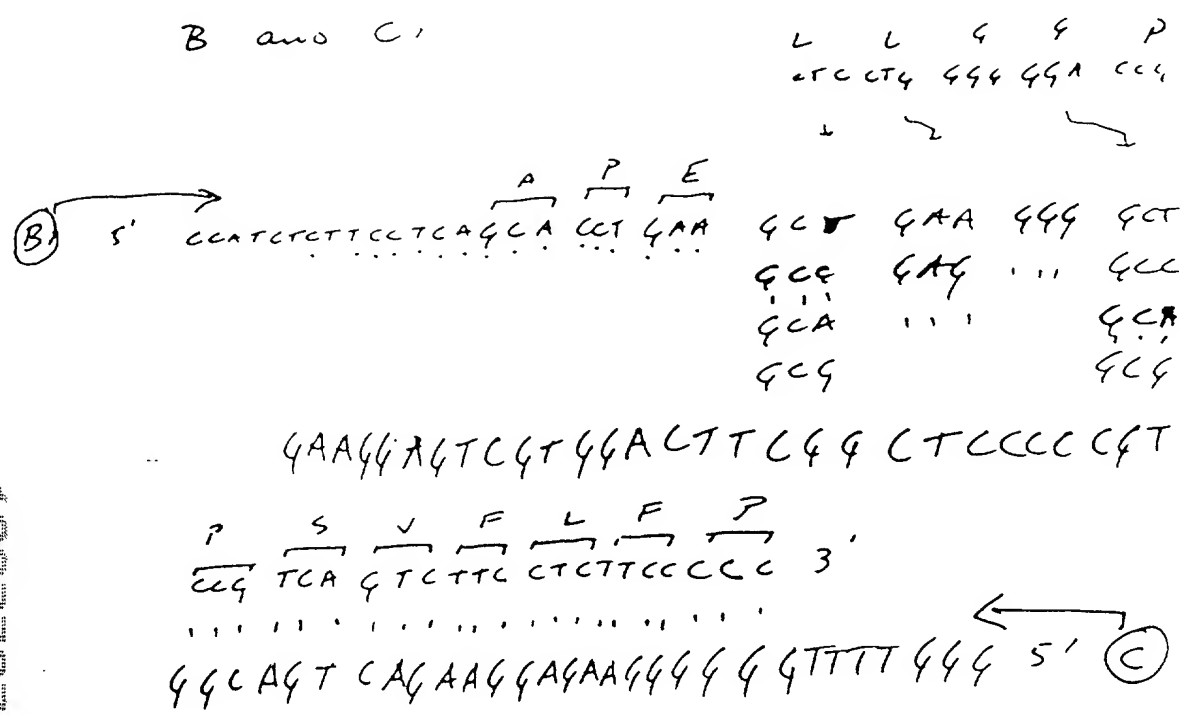
lock

Read and understood by me

Date

A-7

B and C:



Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Bonnicat

PROJECT CHARGED B7 # 967

DATE REQUESTED .

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGAMMA4 - 5'

LENGTH 67

SEQUENCE:

pdT

5' GAGCATTTTCCTGATCAGGA
GTCCAAATATGGTCCCCATT
CCCCATTCATCCCCAGGGTAAG
CCAAACCC 3'

Read and understood by me:

Date

A-8

Transient Expression of IgLhCTLA4(3) Ig /EE12

→ 38

293 culture supernatant tested again a IgG1, IgG4 ELISA. using higher dilution.

Results:

DATE:

293 Transients

		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4 (P2) - γ1	1	2.12	1.77	↓
CTLA4 - mγ4	2	14.88	3.23	
IgL CTLA4 (-2) - γ1	3	34.26	33.65	
IgL CTLA4(3) - γ1	4	33.9	35.54	

⊕ Controls 2.25 μg/ml 156 ng/ml

expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 bin. Assay run by Nancy Green.

Optical Density

		1 (unlabelled CTLA4 Ig)	2 (#1)	3 (#2)	4 (#3)	5 (#4)	6 (WT)	7	8	9	10	11	12
70 ng/ml	A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25	B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5	C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25	D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
15.6	E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8	F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9	G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0	H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max signal - no inhibitor)

As before the IgLLCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete w CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NS0 lines.

→ Samples titrated serially 1:2 - in 50 uL

→ All sample wells contain 50 of 70ng/ml CTLA4 Ig biotin

→ 43

Read and understood by me Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver

Group Art Unit: 1642

Examiner: Helms, L.

NEW Serial No.: 09/227,595 Continuation of

Filed: ~~January 8, 1999~~ December 20, 2001

For: CTLA4-Cγ4 Fusion Proteins (as amended)

Attorney Docket No.: RPN-001

Assistant Commissioner for Patents
Washington, D.C. 20231

NEW Certificate ~~of First Class Mailing~~ (37 CFR ~~1.8(a)~~ Under 1.10)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ Express Mail To Addressee in an envelope addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

By: Larry Taylor
~~Megan E. Williams~~ Larry Taylor
Registration No. ~~43,270~~
~~Attorney for Applicants~~ NEW

Mailing Label No. EL833139174US

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

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Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

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Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
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These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

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Date: _____ Signed: _____

Gary S. Gray

Date: _____ Signed: _____

Jerry Carson

Date: 10-3-01 Signed: Kashi Javaherian

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: _____ Signed: _____

Sandra Silver

HUMAN - CD44 : Ig

STATUS : This clone, which is currently producing large amounts of soluble protein in CHO DG44, is being methotrexate amplified.

RECENTLY focus was shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Caulfield + Morrison, 1991 J Exp Med (173) 4
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see p 115, Book 1139 and //

see p 52, Book 1177 and //

Read and understood by me

Date

Scott M. Cain

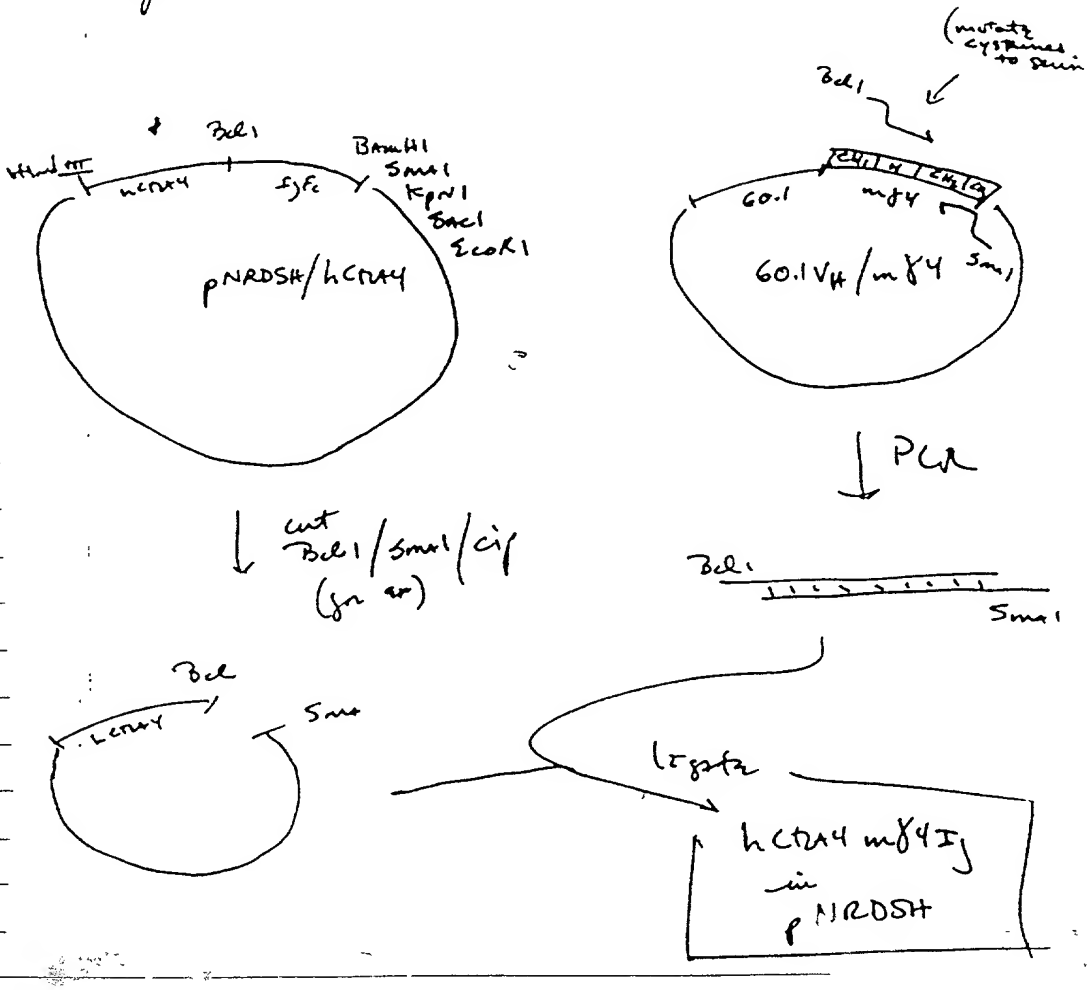
2 STRATEGIES will be USED:

hCγ4, mutagenesis of FcγR

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the EXISTING γ_1 H-CH_2-CH_3

(Note that γ4 also lacks any ability to activate complement - S. Silver)

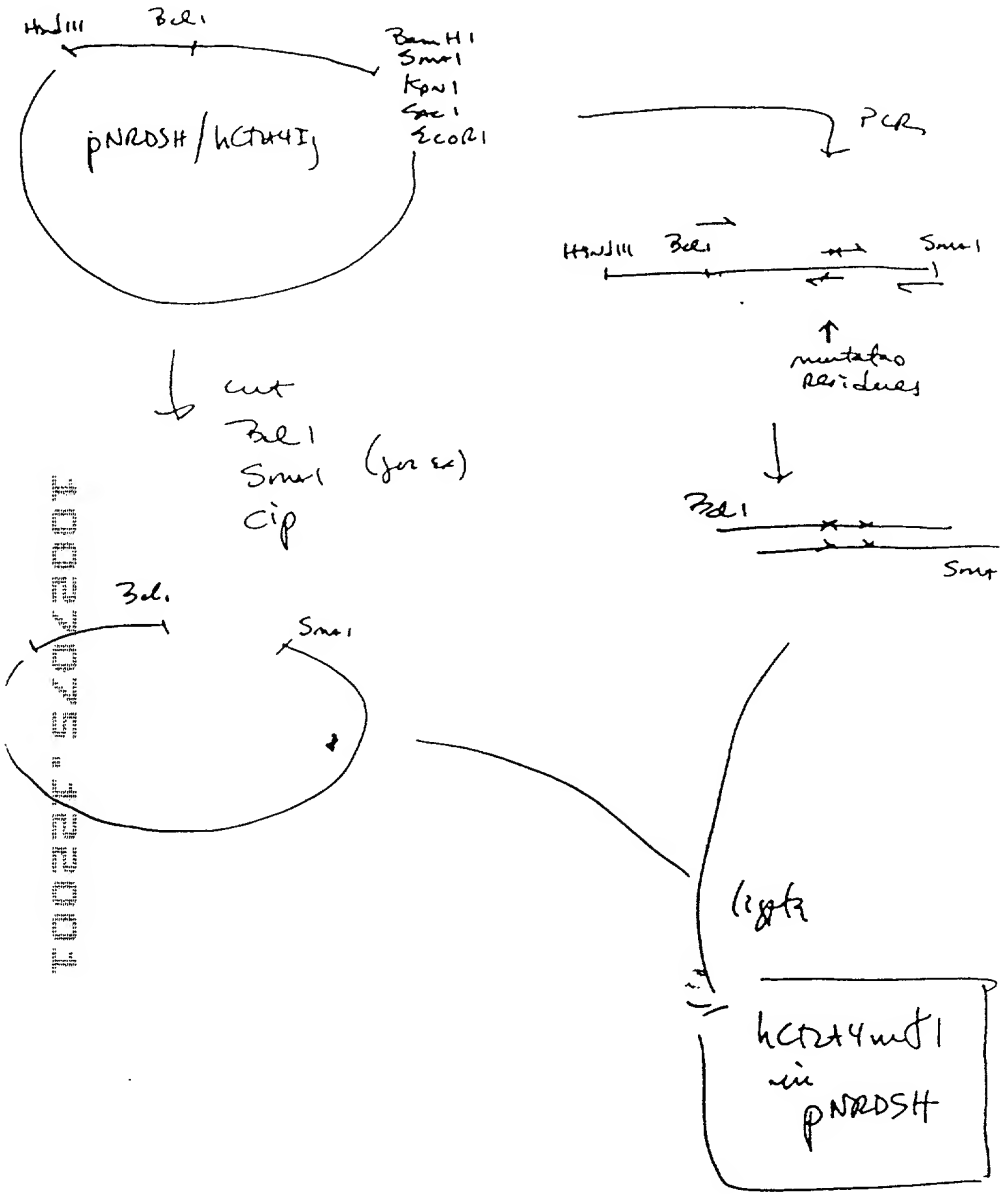


Read and understood by me

Date

A-3

USE NESTED PCR to gener - a mutated 81 from hCTLA4 Ig. Clone the m81 back into hCTLA4 ___ M
pNRDSH:



For this clone mutate residues as follows:

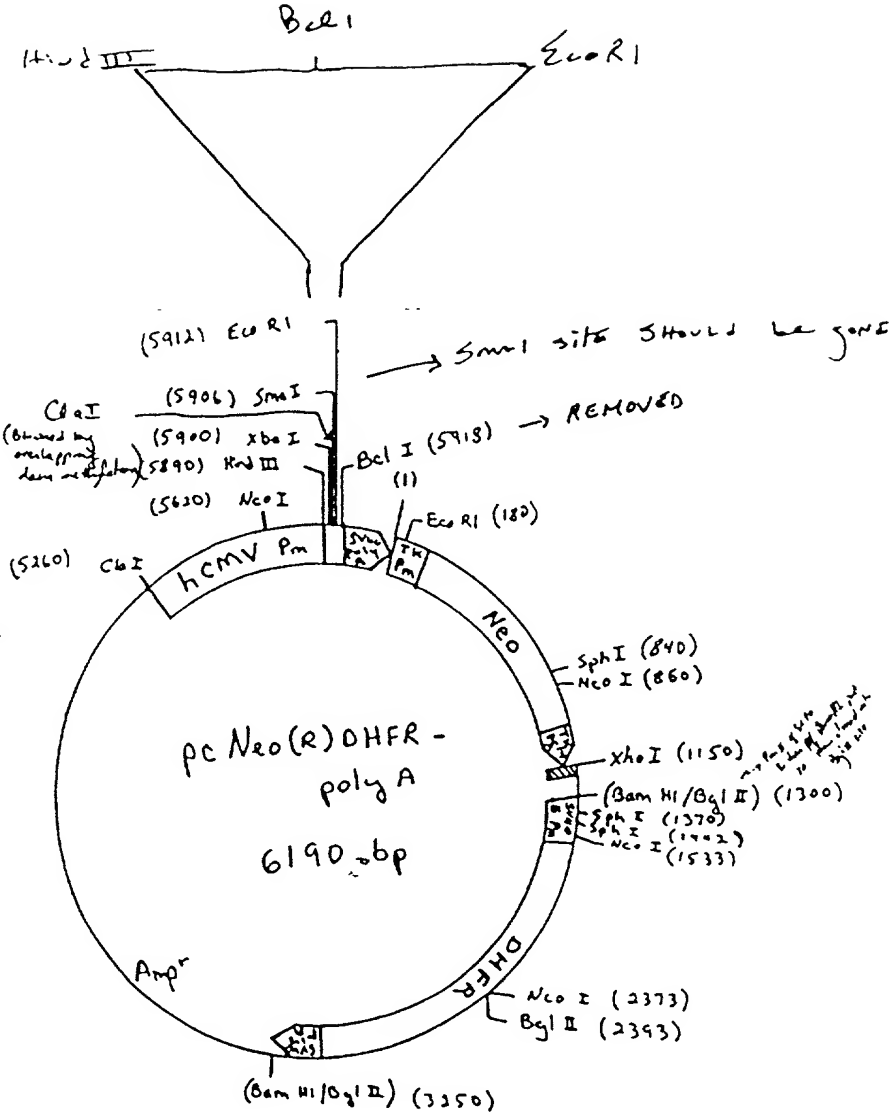
234 L → A
235 L → E
236 G
237 G → A

Read and understood by me

Date

[signature]

Vector:



preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (9-9-92)

Read and understood by me

Date

primers for site mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

```
                    P    D (Q)                    E    S    K    Y
                        BCL 1
5'  GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT
      G    P    P    S    P    S    S    P
    GGT CCC CCA TCC CCA TCA TCC CCA
```

γ4 sequence: (G) (K) (P) (T)
GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

3' primer

1st use this

if needed still have these →

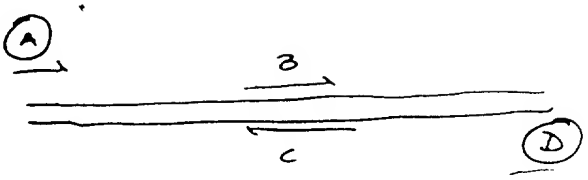
```
5'  GCAGAG GAATTC GAG CTC GGTACCC GGGG ATCC
    lock    R1      Sac1    KpN1   Xma1   BamH1
```

γ4 sequence: CCAGTGTGGGG ACAG TGGG A CC CGCTCT GCC TCCC 3'

Read and understood by me

Date

Donald R Cox



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT

H T S P P S P G K

CAC ACA TCT CCA CCG TCT CCA GGT AAA C — $Ig F_c$ —

— * — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-Bgl2 —

— T7 promoter

3' primer (D): XmaI

BamHI SmaI KpnI SacI EcoRI 3'

pSR72 MCS: 5' GGATCCCC GGGTACC GAG CTC GA ATTC

3' CCTAGGGG CCCATGG CTC GAG CTTAAG 5'

PRIMER:

5' GCAGAG GAATTCGAG CTC GGTACCCGGGGATCC

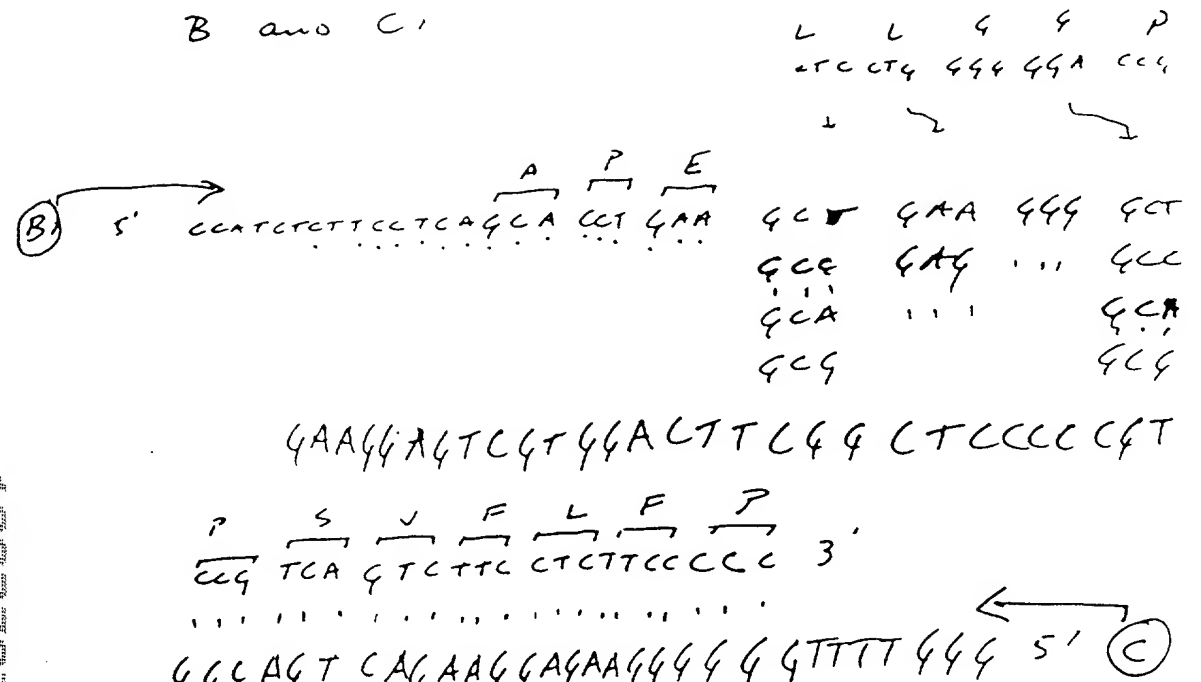
lock

Read and understood by me

Date

A-7

B and C:



Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rowcroft

PROJECT CHARGED 87 # 967

DATE REQUESTED

DATE REQUIRED (NO ASAP)

SEQUENCE NAME MuGamma4 - 5'

LENGTH 67

SEQUENCE:

pol I

5' GAGCATTTTCCTGATCAGGA
GTCCAAATATGGTCCCCATT
CCCCATCATTCCCCAGGTAAG
CCAAACC 3'

Read and understood by me

Date

Transient Expression of IgL hCTLA4(F3) Ig fusion

→ 35

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Result:

DATE:

293 TRANSIENTS

		ug/mL IgG1	ug/mL IgG4	Dilutions 1:10 → 1:2
IgL CTLA4(F2)-γ1	1	2.12	1.77	
~~mCD~~ CTLA4-mγ4	2	14.88	3.23	
IgL CTLA4(-2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.91	35.54	

⊕ Control: 2.25 μg/mL 156 ng/mL

Expected: 2 μg/mL 125 ng/mL

These samples were tested for their ability to compete for B7 binding. Assay run by Nancy Green.

		Unrelated IgG1 CTLA4Ig	IC samples #1	#2	#3	#4	#5	Optical Density WT mCTLA4	WT hCTLA4	mCTLA4 Δ MYPY	hCTLA4 Δ MYPY	mCTLA4 Δ MYPY	hCTLA4 Δ MYPY	11	12
20 μg/mL	A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458				
10	B	0.170	0.412	0.063	0.083	0.075	0.128	0.147	0.182	0.291	0.343				
5	C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318				
2.5	D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398				
1.2	E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381				
0.6	F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415				
0.3	G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408				
0	H	0.425	0.849	0.407	0.452	0.524	0.597	0.496	0.489	0.523	0.424				

(max wavelength signal - background)

→ Samples titrated serially 1:2 - in 50T vol

→ All sample wells contain 50T of 70ng/ml CTLA4Ig human

As before the IgL CTLA4 is not functional. The two clones of IgL hCTLA4 do effectively compete CTLA4-Ig - 2:1 molar ratio.

Plasmids are ready for transfection into stable NSO lines.

→ 43

Read and understood by me Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver

Serial No.: 09/227,595 Continuation of

Filed: ~~January 8, 1999~~ December 20, 2001

For: CTLA4-Cγ4 Fusion Proteins (as amended)

Attorney Docket No.: RPN-001

Group Art Unit: 1642

Examiner: Helms, L.

Assistant Commissioner for Patents
Washington, D.C. 20231

Certificate ~~of First Class Mailing~~ (37 CFR ~~1.8(a)~~ Under 1.10)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ "Express Mail to Addressee" in an envelope addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

Mailing Label No. EL 933315914 US

By: Larry Taylor
~~Megan E. Williams~~
~~Registration No. 43,270~~
~~Attorney for Applicants~~

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

2. Prior to July 11, 1994, the invention described and claimed in the above-referenced application was completed in this country, as evidenced by the following:

A. Prior to July 11, 1994, we were in possession of a cloned CTLA4Ig molecule which was producing large amounts of active protein in CHO DG44 cells.

Page A-1 of Appendix A describes amino acid modifications to the Ig portion of the CTLA4Ig molecule. Deletion of the CH_2 domain from γ1 and mutations to amino acids 235 and 237 in γ4 are described.

Page A-2 of Appendix A describes amplification of the mutated γ4 Hinge-CH_2-CH_3 region and the cloning of the mutated γ4 into pNRDSH/hCTLA4 to replace the existing γ1 Hinge-CH_2-CH_3.

Page A-3 of Appendix A describes a second method which involves the use of nested PCR to generate a mutated γ1 (having an L to A substitution at amino acid 234, an L to E substitution at amino acid 235, and a G to A change at amino acid 237) from hCTLA4Ig and the cloning of the mutated γ1 back into hCTLA4 pNRDSH.

Pages A-5 and A-6 show primers for use in making mutated forms of CTLA4Ig.

The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
In the experiment presented at the bottom of the page B7Ig was put onto plates. Biotinylated CTLA4Ig was added to the plates in a fixed amount. Test forms of CTLA4Ig were added in serial dilutions to test for their ability to compete for binding to B7 on the plate. In this type of assay as the amount of an effective competitor is increased, the amount of biotinylated CTLA4Ig that binds decreases. This leads to a decrease in the optical density readout. Unlabelled CTLA4Ig (left row) was used as a positive control.
The data show that samples #2, #3, and #4 (in rows 2, 4, and 5, respectively) effectively competed with unmodified CTLA4Ig for binding to B7. As indicated in the table in the middle of the page, sample 2 was oncoCTLA4-mγ4; sample 3 was IgLCTLA4-γ1; and sample 4 was IgLCTLA4-γ1.

These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

Each of the dates deleted from pages A-1 through A-8 of Appendix A are prior to July 11, 1994.

We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: ____________________ Signed: ____________________

Gary S. Gray

Date: ____________________ Signed: ____________________

Jerry Carson

Date: ____________________ Signed: ____________________

Kashi Javaherian

Date: 3 October 2001 Signed: [signature]

Paul D. Rennert

Date: ____________________ Signed: ____________________

Sandra Silver

HUMAN - CD44 : Ig

STATUS : This clone, which is currently producing large amounts of active protein in CHO DG44, is being methotrexate amplified.

Recently focus has shifted to the Ig tail portion of this clone. Both FC receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
Jung et al, 1991 J Immunol. (147)
Tao et al, 1991 J Exp Med (173) 1025
Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from γ_1 and mutated residues 235 and 237 in γ_4

See p 115, Book 1139 and //

See p 52, Book 1177 and //

Read and understood by me

Date

/Scott M. Cain

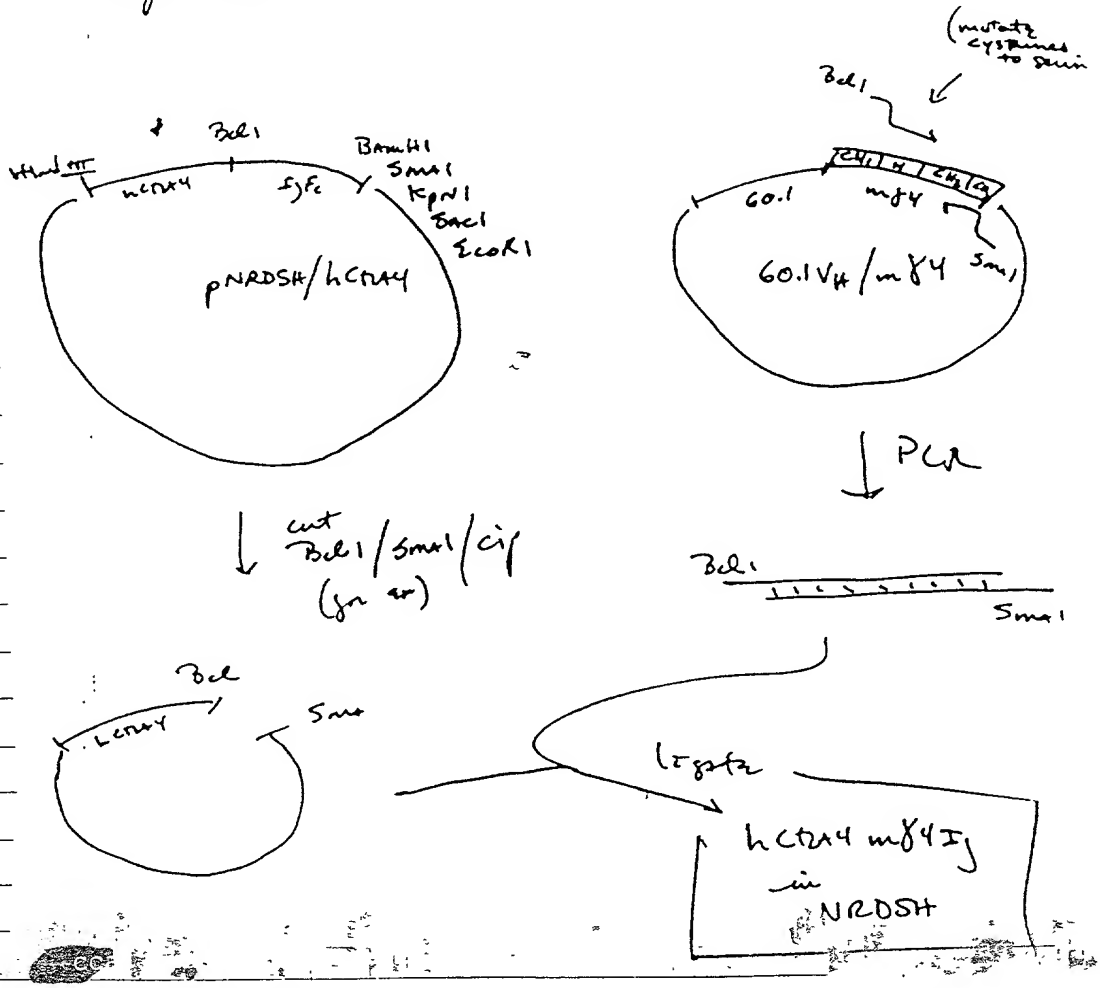
2 STRATEGIES will BE USED:

hCγ4, mutagenesis of IgFc

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the existing γ_1 H-CH_2-CH_3

(Note that γ4 also lacks any ability to activate complement - S. Silver)

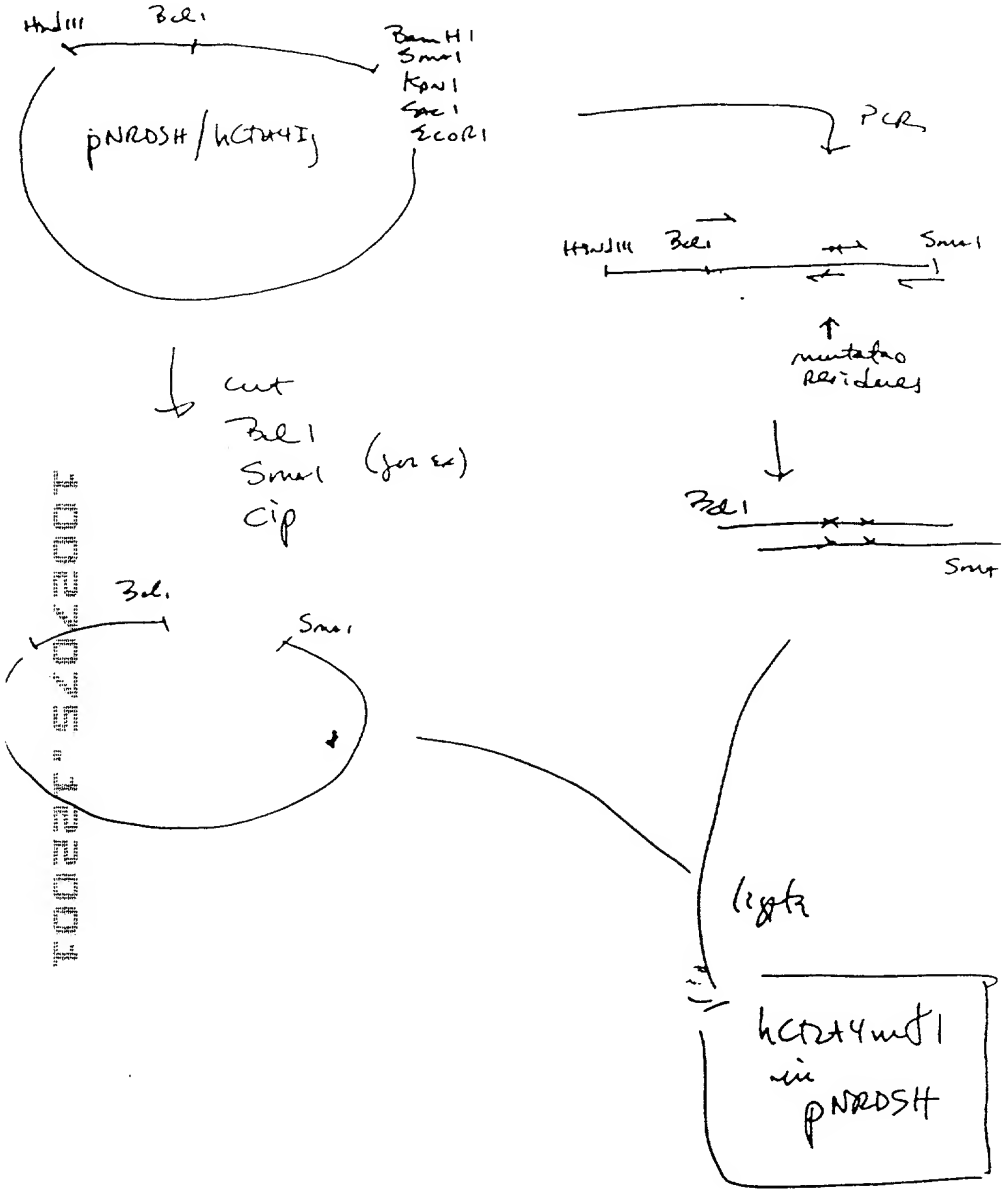


Read and understood by me

Date

USE NESTED PCR to gener - a mutated 81 from hCD24 Ij . Clone the mf1 back into hCD24. M

pNRDSH :



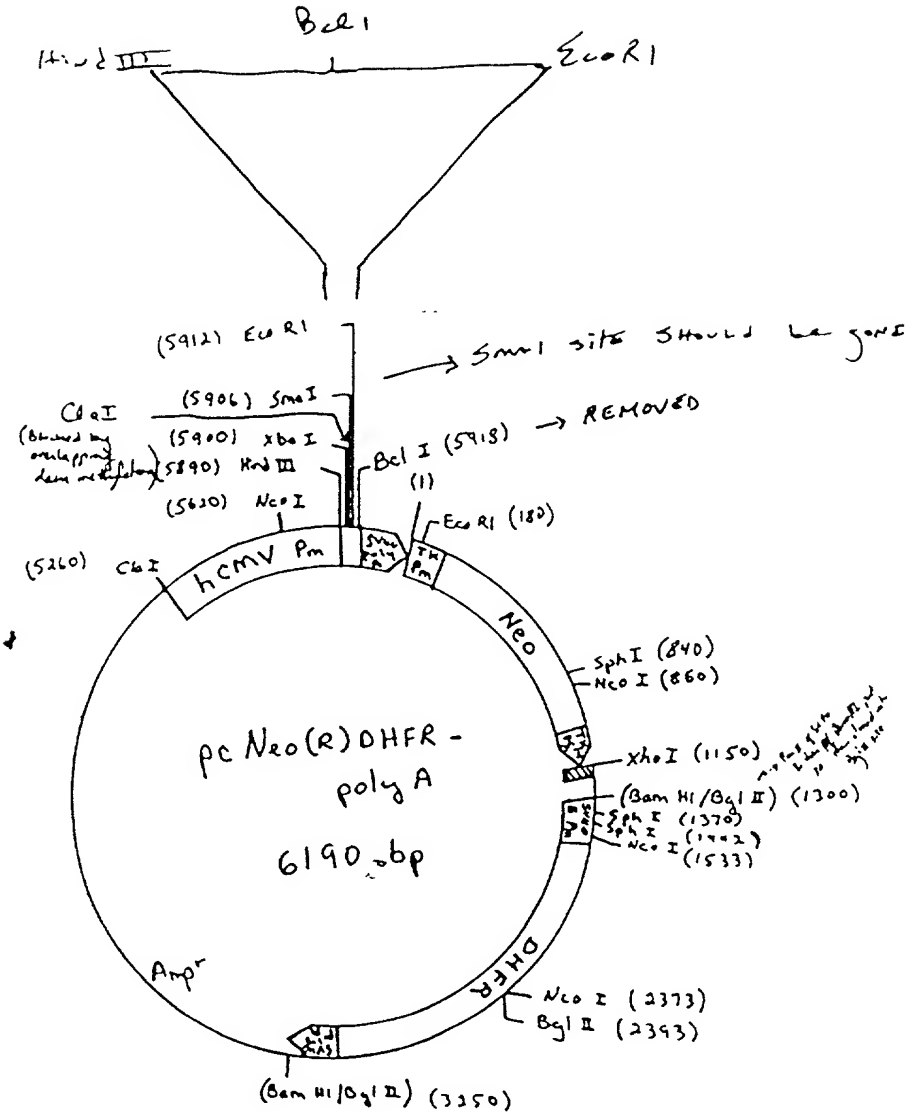
For this clone mutate residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



Enzymes that DO NOT CUT

EcoRV 1227 r3
SpeI 1227 r3
KpnI (4-9-92)

Read and understood by me

Date

primers for -site mutagenesis

for γ4:

5' primer - use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

(G) (K) (P) (T)

GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

3' primer

1st use this

if needed still have these →

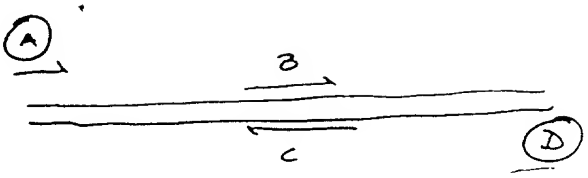
5' GCAGAG GAATTC GAG CTC GGTACCC GGGGATCC

lock R1 Sac1 KpN1 Xma1 BamH1

4 me CCAGTGTGGGG ACAG TGGG A CC CGCTCT GCC TCCC 3'

Read and understood by me Date

Brad A Cass



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT

CAC ACA TCT CCA CCG TCT CCA GGT AAA C — IgF_c —

— * — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-Bgl2 —

— T7 promoter

3' primer (D):

XmaI

BamHI SmaI KpnI SacI EcoRI 3'

pSP72 MCS: 5' GGATCCCC GGGTACC GAG CTC GAATTC

3' CCTAGGGG CCCATGG CTC GAG CTTAAG 5'

PRIMER:

5' GCAGAG GAATTCGAG CTG GGTACCCGGGGATCC

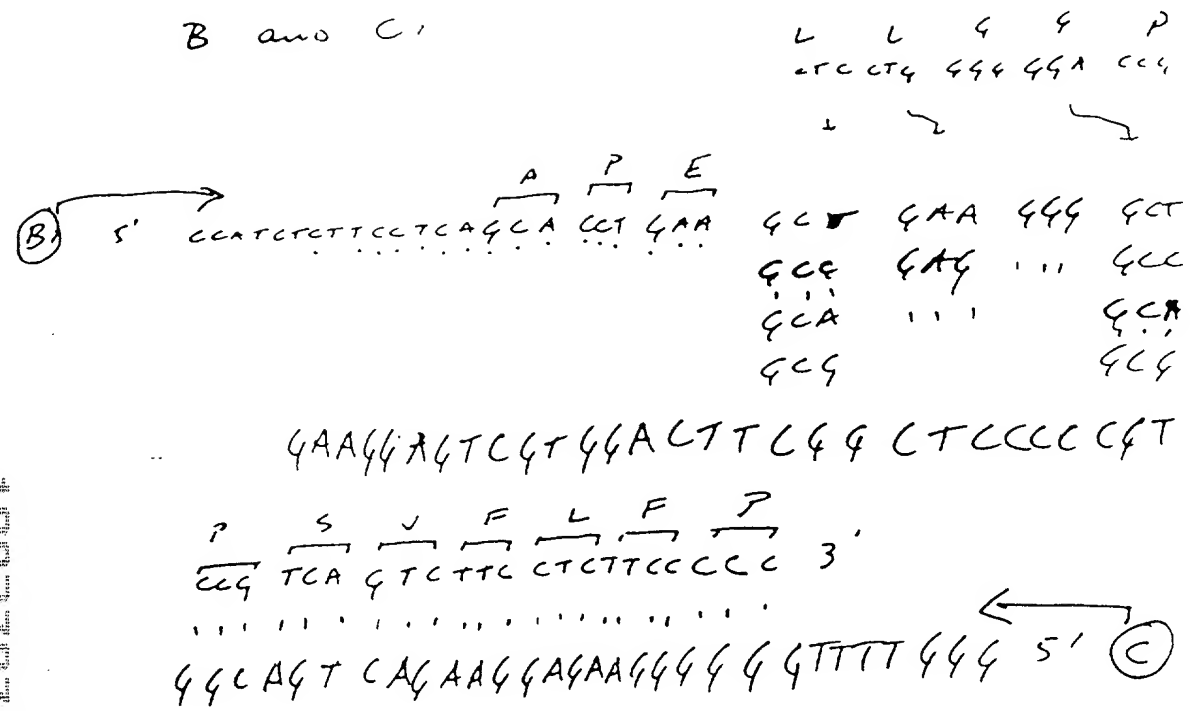
lock

Read and understood by me

Date

A-7

B and C'



Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rowbottom

PROJECT CHARGED B7 # 967

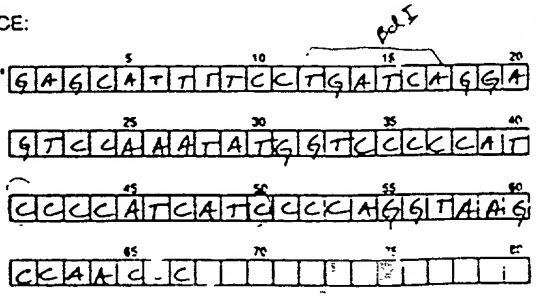
DATE REQUESTED .

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGamma4 - 5'

LENGTH 67

SEQUENCE:



Read and understood by, me

Date

Transient Expression of IgLhCTLA4(3) Ig /EEI2 A-8

→ 3F

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

	ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
IgL (#2) CTLA4-81 1	2.12	1.77	↓
onco CTLA4-m84 2	14.88	3.23	
IgLCTLA4 (-2) -81 3	34.26	33.65	
IgL CTLA4(3)-81 4	33.9	35.54	

⊕ Controls 2.25 μg/ml 156 ng/ml
expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 bin. Assay run by Nancy Green.

Optical Density

	1 (unlabeled CTLA4 Ig)	2 (#1)	3 (#2)	4 (#3)	5 (#4)	6 (WT 6A/11 6/16)	7 (7/13)	8 (WT APTUL 7/13)	9 (A4ng A304)	10	11	12
50 μg/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
15.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max nonlimiting signal — no inhib.)

As before the IgLhCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete w CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NSO line.

→ Samples titrated serially 1:2 – in 50 μL

→ All sample wells contain 50 μL of 70 ng/ml CTLA4 Ig biotin

→ 43

Read and understood by me Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert and Sandra Silver Serial No.: Continuation of 09/227,595 Filed: ~~January 8, 1999~~ December 20, 2001 For: CTLA4-Cγ4 Fusion Proteins (as amended) Attorney Docket No.: RPN-001	Group Art Unit: 1642 Examiner: Helms, L.

Assistant Commissioner for Patents
Washington, D.C. 20231

Certificate ~~of First Class Mailing~~ (37 CFR ~~1.8(a)~~ 1.10 under)

I hereby certify that this correspondence is being deposited with the United States Postal Service as ~~first class mail~~ "Express Mail to Addressee" in an envelope addressed to: ~~Assistant~~ Commissioner for Patents, Box Patent Application, Washington, D.C. 20231 on the date set forth below.

December 20, 2001
Date of Signature and of Mail Deposit

Mailing Label No. EL833315914 US

By: Larry Taylor
~~Megan E. Williams~~ Larry Taylor
~~Registration No. 43,270~~
~~Attorney for Applicants~~

DECLARATION UNDER 37 C.F.R. 1.131 BY

GARY S. GRAY, JERRY CARSON, KASHI JAVAHERIAN, PAUL D. RENNERT AND SANDRA SILVER

Sir:

We, Gary S. Gray, Jerry Carson, Kashi Javaherian, Paul D. Rennert And Sandra Silver declare:

1. We are the inventors of the subject matter described and claimed in the above-referenced patent application.

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The notebook pages submitted as pages A-1 through A-7 of Appendix A demonstrate that we completed the claimed invention in this country prior to July 11, 1994.

B. In addition, as shown on page A-8 of Appendix A, prior to July 11, 1994, we demonstrated that mutated forms of CTLA4Ig effectively competed with biotinylated CTLA4-Ig for binding to B7.
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These data demonstrate that the modified CTLA4Ig molecules were still able to bind to B7.

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We have been warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 or the United States Code, and that such willful and false statements may jeopardize the validity of the subject application or any patent resulting therefrom, and declare that all statements made of our own knowledge are true and that all statements made on information and belief are believed to be true.

Date: _____ Signed: _____

Gary S. Gray

Date: _____ Signed: _____

Jerry Carson

Date: _____ Signed: _____

Kashi Javaherian

Date: _____ Signed: _____

Paul D. Rennert

Date: October 16, 2001 Signed: Sandra Silver

Sandra Silver

HUMAN - CD64 : Is

STATUS : This clone, which is currently producing large amounts of active protein in CHO DG44, is being methotrexate amplified.

RECENTLY focus was shifted to the Ig tail portion of this clone. Both Fc receptor and complement activation activities are determined by sequence in CH_2 domain.

REFS: Canfield + Morrison, 1991 J Exp Med (173) 1483
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Duncan + Winter, 1988 Nature (332)

It would be useful to design a construct in which these Ig activities were eliminated.

This work will be exactly analogous to what was done last year for the antibody engineering project, where I deleted the CH_2 domain from δ_1 and mutated residues 235 and 237 in δ_4

see p 115, Book 1139 and //

see p 52, Book 1177 and //

Read and understood by me

Scott M. Cain

Date

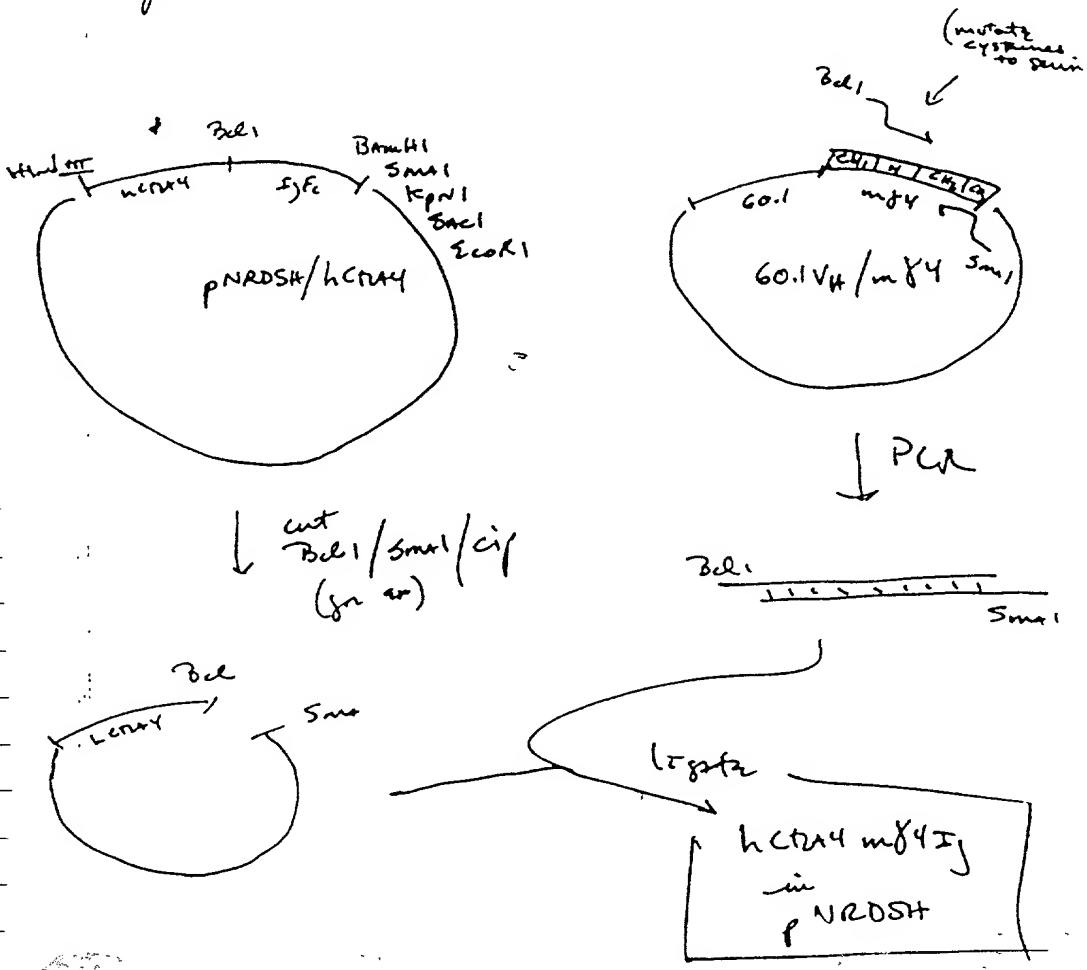
2 STRATEGIES will be USED:

hCγ4, mutagenesis of γ_1Fc

possible STRATEGIES:

① PCR out the mutated γ4 H-CH_2-CH_3 region from 60.1 V_H and clone into pNRDSH/hCγ4 in place of the EXISTING γ_1 H-CH_2-CH_3

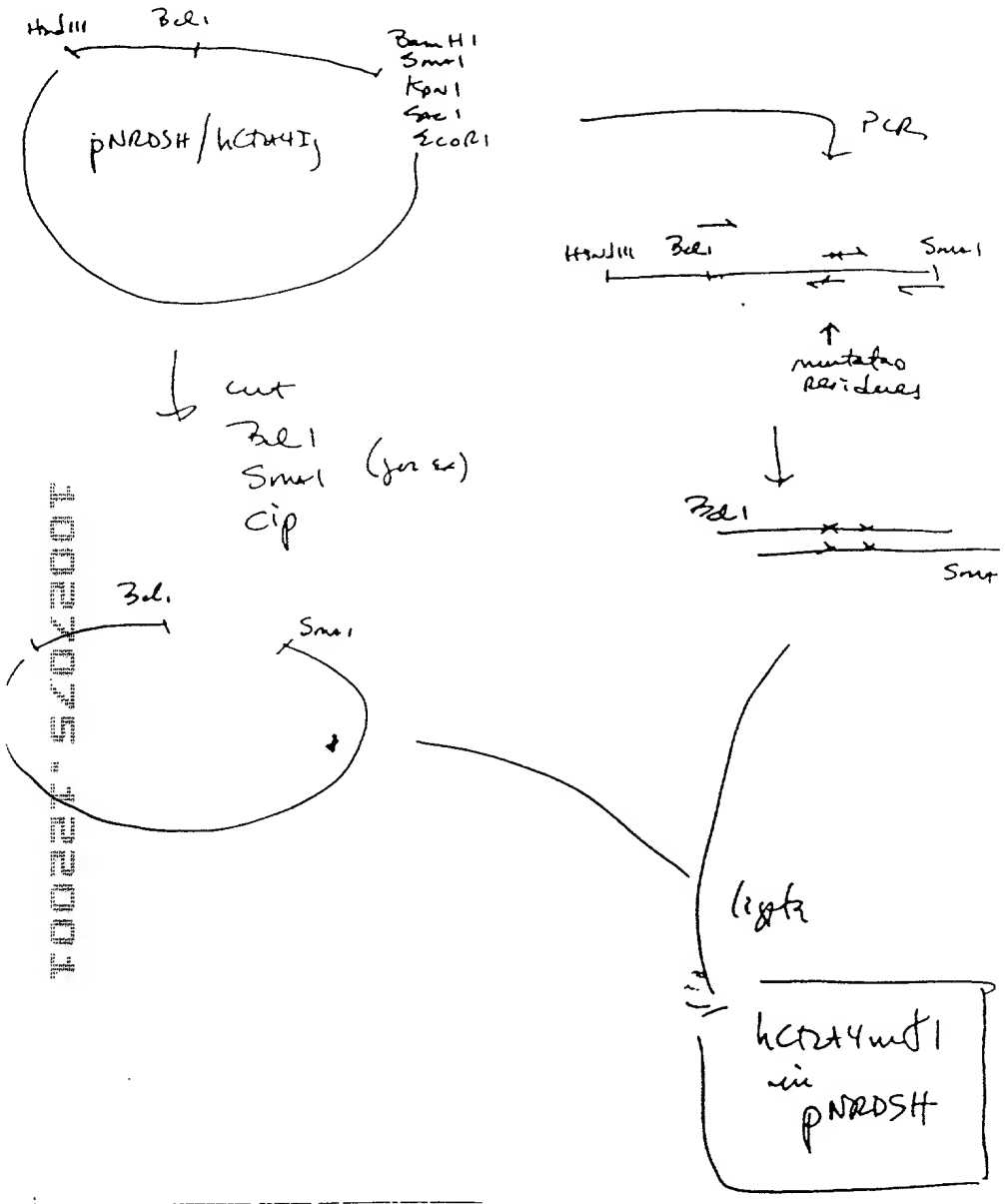
(Note that γ4 also lacks any ability to activate complement - S. Silver)



Read and understood by me

Date

USE NESTED PCR to gener - a mutated 81 from hC1214Ij . Clone the mf1 back into hC1214. M
pNRDSH :



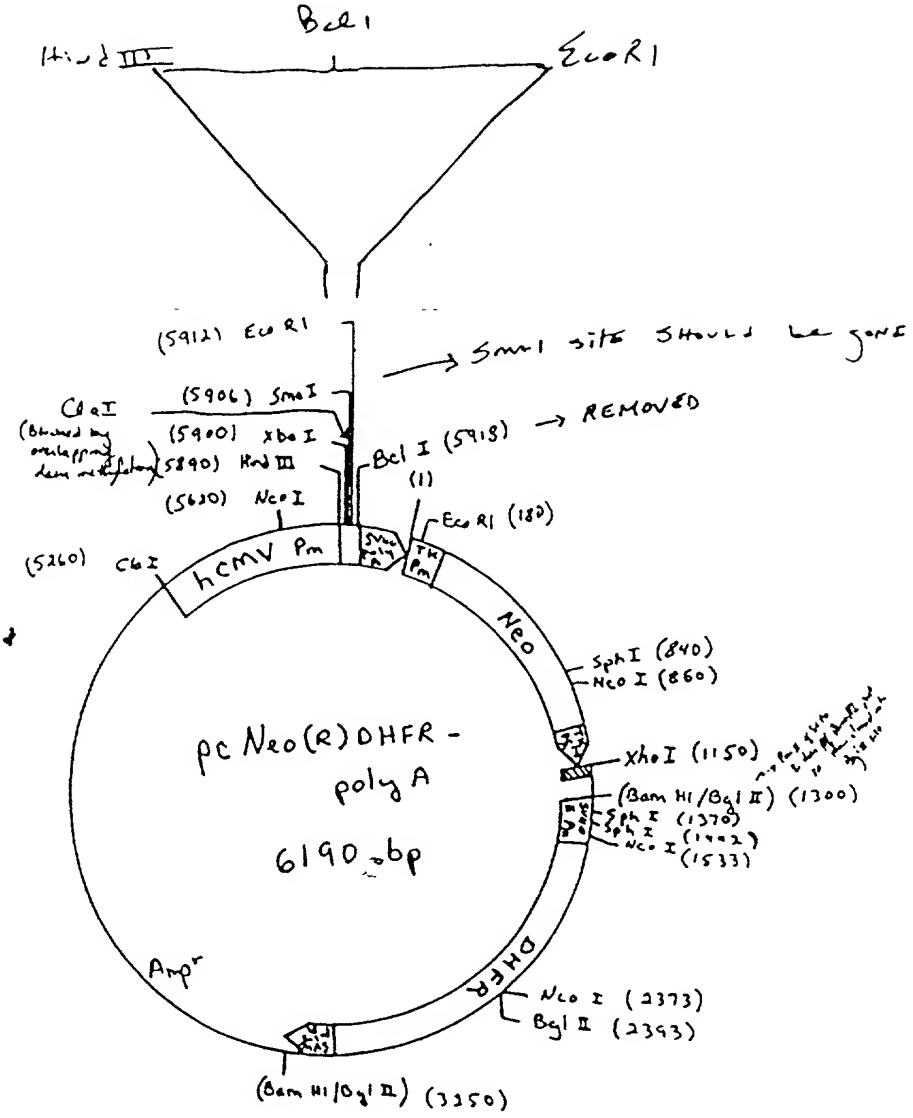
For this clone mutate residues as follows:

234	L	→ A
235	L	→ E
236	G	
237	G	→ A

Read and understood by me

Date

Vector:



▨ preproinsulin poly A

Enzymes that DO NOT CUT

EcoRV 1227 p3
SpeI 1227 p3
KpnI (4-9-92)

Read and understood by me

Date

primers for site-directed mutagenesis

for γ4:

5' primer – use G. Gray's original idea to knock out the cysteines in the hinge (γ4 has two)

P D (Q) E S K Y

BCL 1

5' GAG CAT TTT CCT GAT CAG GAG TCC AAA TAT

G P P S P S S P

GGT CCC CCA TCC CCA TCA TCC CCA

primer → (G) (K) (P) (T)

γ4 GGT AAG CCA ACCC

DOUBLE CHECK THAT pNRDSH LACKS THIS restriction site

3' primer

1st use this

if needed still have these → →

5' GCAGAG GAATTC GAG CTC GGTACCC GGGGATCC

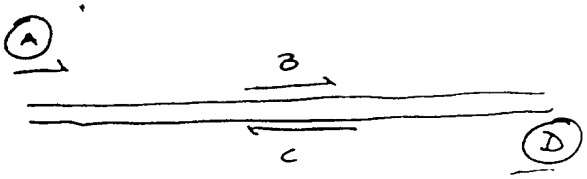
lock R1 Sac1 KpN1 XmaI BamHI

γ4 primer CCAGTGTGGGG ACAG TGGG A CC CGCTCT GCC TCCC 3'

Read and understood by me

Date

Scott R Case



5' primer ✓

(A): use Gary Gray's original γ_1 primer:

PRIMER

5' GAG CAT TTT CCT GAT CAG GAG CCC AAA TCT TCT CAC AAA ACT
P P BglII Q E P K S S D K T
CAC ACA TCT CCA CCG TCT CCA GGT AAA C — $Ig F_c$ —
H T S P P S P G K

— * — BamHI-SmaI-KpnI-SacI-EcoRI-ClaI-EcoRS-BglII —

— T7 promoter

3' primer (D): XmaI

BamHI SmaI KpnI SacI EcoRI 3'

pSR72 MCS: 5' GGATCCCC GGGTACC GAGCTC GAATTC

3' CCTAGGGG CCCATGG CTCGAG CTTAAG 5'

PRIMER:

5' GCAGAGGAATTCGAG CTCGGTACCCGGGGATCC

lock

Read and understood by me

Date

A-7

B and C

L L G G P
CTC CTG GGG GGA CCG

A P E
(B) 5' CCATCTCTTCCTCAGCA CCT GAA GCT GAA GGG GCT
GCG GAG GCC
GCA GCA
GCG GCG

GAAGGAGTCGTGGACTTCGG CTCCCCGT

P S V F L F P
CCG TCA GTCTTC CTCTTCCCCC 3'

GGCAGT CAGAAGGAGAAGGGG GGTTTT GGG 5' (C)

Oligonucleotide Requests:

DNA SYNTHESIS REQUEST FORM

REQUESTED BY Paul Rennert

PROJECT CHARGED 37 # 9GT

DATE REQUESTED

DATE REQUIRED (NO ASAP)

SEQUENCE NAME muGamma4 -5'

LENGTH 67

SEQUENCE:

BclI

5' GAGCATTTTCCTGATCAGGA
GTCCAAATATGGTCCCCCAT
CCCCATCATCCCCAGGTAAG
CCAACCC 3'

Date

Read and understood by me

[signature]

Transient Expression of IgLhCTLA4(3) Ig / EE12

→ 38

293 culture supernatant tested again a IgG1, IgG4 ELISA using higher dilution.

Results:

DATE:

293 Transients

		ug/mL IgG 1	IgG 4	Dilutions 1:10 → 1:2
ILL CTLA4(+2)-γ1	1	2.12	1.77	↓
CTLA4-mγ4	2	14.88	3.23	
IgL CTLA4(-2)-γ1	3	34.26	33.65	
IgL CTLA4(3)-γ1	4	33.9	35.54	

⊕ Controls 2.25 μg/ml 156 ug/ml
expected: 2 μg/ml 125 ng/ml

These samples were tested for their ability to compete for B7 bin. Assay run by Nancy Green.

	1 (unlabelled CTLA4Ig)	2 (JC samples #1)	3 (#2)	4 (#3)	5 (#4)	6 (WT)	7	8	9	10	11	12
50 ug/ml A	0.119	0.404	0.078	0.066	0.063	0.134	0.128	0.150	0.253	0.458		
25 B	0.170	0.412	0.083	0.083	0.075	0.128	0.147	0.182	0.291	0.343		
12.5 C	•0.209	0.349	0.096	0.090	0.076	0.153	0.192	0.173	0.323	0.318		
31.25 D	0.287	0.412	0.138	0.104	0.096	0.199	0.237	0.233	0.448	0.398		
15.6 E	0.342	0.450	0.157	0.144	0.122	0.260	0.288	0.282	0.445	0.381		
7.8 F	0.390	0.454	0.268	0.158	0.149	0.285	0.368	0.349	0.549	0.415		
3.9 G	0.384	0.504	0.279	0.198	0.183	0.369	0.462	0.425	0.392	0.408		
0 H	0.425	0.649	0.407	0.462	0.524	0.597	0.496	0.489	0.523	0.424		

(max signal – no ...)

As before the IgLhCTLA4 is not functional. The two clones of IgLhCTLA4 do effectively compete with CTLA4-Ig-biotinylated.

Plasmids are ready for transfection into stable NS0 lines.

→ Samples titrated serially 1:2 – in 50 uL
→ All sample wells contain 50 of 70 ng/ml CTLA4Ig biotin

→ 43

Read and understood by me   Date

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Sequence Listing page number starts on page 52.